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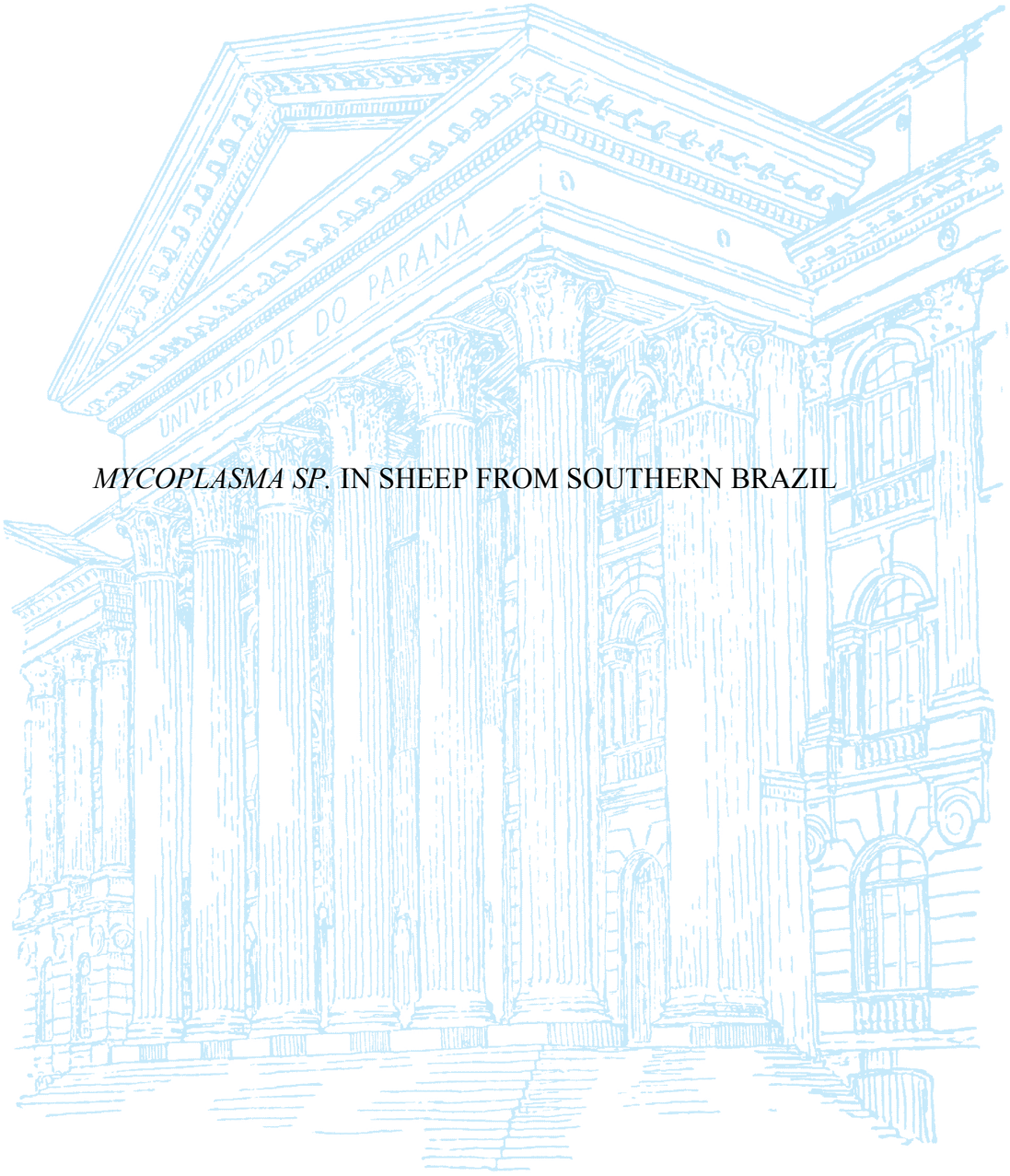
ANNA CLAUDIA BAUMEL MONGRUEL



MYCOPLASMA SP. IN SHEEP FROM SOUTHERN BRAZIL

CURITIBA

2019

ANNA CLAUDIA BAUMEL MONGRUEL

Mycoplasma sp. in sheep from southern Brazil

Dissertação apresentada ao curso de Pós-Graduação em Ciências Veterinárias, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito à obtenção do título de Mestre em Ciências Veterinárias.

Orientador: Prof. Dr. Rafael Felipe da Costa Vieira

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS VETERINÁRIAS da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **ANNA CLAUDIA BAUMEL MONGRUEL** intitulada: ***Mycoplasma* sp. in sheep from southern Brazil**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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“I don’t know where I’m going from here. But I promise it won’t be boring”

David Bowie

RESUMO

O rebanho mundial de ovinos é estimado em mais de um bilhão de animais e a América do Sul representa 6,8% da produção mundial de ovinos. Embora as ovelhas sejam criadas principalmente para fins de obtenção de carne, esses animais fornecem uma ampla gama de produtos, como leite, pele e lã. Em 2016, o rebanho ovino brasileiro atingiu o número estimado de 18.000.000 de animais somado a um crescente cenário de produção e consumo de produtos ovinos à longo prazo. O Paraná, localizado na região sul do país, possui um rebanho estimado em mais de 500.000 cabeças, representando 3,12% do rebanho nacional e considerado um negócio em crescimento. Diversos hemoparasitos, como bactérias e protozoários, são descritos infectando ovinos de todo o mundo, o que pode causar fases febris, anorexia, anemia e perdas econômicas envolvendo esses animais. O presente estudo tem como objetivo identificar o agente causador de um surto de anemia em um rebanho ovino do município de Bandeirantes, Paraná, sul do Brasil. Quarenta e seis ovinos mantidos no município de Bandeirantes, região norte do Paraná, foram avaliados molecularmente por PCR quanto à presença de hemoplasmas, piroplasmas e *Anaplasma* spp. A infecção por parasitas gastrintestinais foi avaliada pela contagem de ovos por grama (OPG). Coleta de carrapatos, avaliação hematológica e análise filogenética também foram realizadas. Todas as amostras foram negativas para a presença de material genético de *Anaplasma* spp. e piroplasmas. Sete amostras (16,6%) apresentaram amplificações do tamanho esperado dos *primers* utilizados para detecção de *Mycoplasma* spp. Três amostras foram escolhidas para sequenciamento e apresentaram similaridade de 99% com sequências dos fragmentos 16S rDNA de *M. ovis* depositados no banco de dados do GenBank. A análise de haplótipos demonstrou a presença de três genótipos diferentes. Dentre 38 animais avaliados, 24 (63,15%) apresentaram valores de EPG acima de 500 e foram considerados positivos para a presença de ovos do tipo Strongylida. Carrapatos da espécie *Rhipicephalus sanguineus* foram coletados de dois animais. Na análise filogenética, as sequências do presente estudo foram alocadas em um clado conjunto com sequências de *Mycoplasma* sp. e *Mycoplasma ovis* isoladas em hospedeiros de outras regiões do mundo. O presente estudo relata a ocorrência de *Mycoplasma* sp. em ovinos do estado do Paraná. A co-infecção de parasitas gastrointestinais e hemoplasmas pode agravar a condição de saúde de ovelhas, representando um alerta a cerca do diagnóstico de hemoplasmas em rebanhos de ovelhas onde ocorre infecções por parasitos gastrointestinais.

ABSTRACT

Worldwide sheep herd is estimated in more than one billion animals and South America represents 6.8% from the world sheep production. Although sheep are raised primarily for meat purposes, these animals provide a wide range of products, such as milk, skin and wool. In 2016, the Brazilian sheep herd reached the estimated number of almost 18,000,000 of animals added to an increasing long-term scenario of production and consumption of sheep products. Paraná State, located in the southern region, have an estimated herd of more than 500,000 sheep, representing 3,12% from the national herd and considered a growing business. A wide range of hemoparasites, such as bacteria and protozoa, are described infecting sheep from worldwide, which may cause febrile phases, anorexia, anemia and economic losses in sheep. The present study aims to identify the causative agent of an outbreak of anemia in a sheep herd from Bandeirantes County, Paraná State, southern Brazil. Forty-six sheep maintained at Bandeirantes county, north region of Paraná State, were molecularly evaluated by PCR for the presence of hemoplasmas, piroplasms and *Anaplasma* spp. Infection by gastrointestinal parasites were accessed by worm egg per gram counting (EPG). Collection of ticks, hematological evaluation and phylogenetic analysis were also performed. All samples were negative for the presence of genetic material from *Anaplasma* spp. and piroplasms. Seven samples (16,6%) obtained amplifications of the expected size from the primers used for *Mycoplasma* spp. detection. Three samples were choosing for sequencing and obtained a similarity of 99% with sequences of the 16S rDNA fragments from *M. ovis* deposited in GenBank database. Haplotype analysis showed the occurrence of three different genotypes. From 38 animals evaluated, 24 (63,15%) presented EPG values above 500 and were considered positive for the presence of Strongylida type eggs. *Rhipicephalus sanguineus* ticks were collected from two animals. Sequences from the present study was placed together in a clade with *Mycoplasma* sp. and *Mycoplasma ovis* sequences from worldwide. Herein we report the occurrence of *Mycoplasma* sp. in sheep from Paraná State. Co-infection of gastrointestinal parasites and hemoplasmas may worse health condition of sheep, representing an alert about hemoplasmas diagnosis in ovine herds with nematode infections occurrence.

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PAPER FOR SUBMISSION

LIST OF ABBREVIATIONS

RBCs: Red blood cells

PCR: Polymerase chain reaction

PCV: Packed cell volume

GI: Gastrointestinal

EPG: Eggs per gram

B.C.: Before Christ

TBF: Tick-borne fever

DNA: Deoxyribonucleic acid

FEC: Fecal egg count

rDNA: Ribosomal deoxyribonucleic acid

TBD: Tick-borne diseases

mL: Milliliter

L/L: Liter of cells per liter of blood

EDTA: Ethylenediamine tetra acetic acid

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

bp: Base pair

BIC: Bayesian Information Criteria

CI: Confidence interval

NHP: Non-human primates

uL: Microliter

USA: United States of America

SUMMARY

1 INTRODUCTION

Hemotropic mycoplasmas, also known as hemoplasmas, are small and pleomorphic bacteria of red blood cells (RBCs) that may infect many different species of vertebrate hosts (MESSICK et al., 2004). *Mycoplasma ovis* (formerly Eperythrozoon ovis) is a wall-less bacterium and it has been described infecting sheep (NEIMARK et al., 2004), goats (JOHNSON et al., 2016; MACHADO et al., 2017), wild ruminants (GRAZZIOTIN et al., 2011; GRAZZIOTIN et al., 2011b) and human beings (SYKES et al., 2010). In sheep, clinical manifestations of *M. ovis* infection may include weight loss (MARTINEZ-HERNANDEZ et al., 2018), hyperthermia, mucosal pallor and hemolytic anemia (NEIMARK et al., 2004; AKTAS, OZUBEK, 2017).

Diagnosis of sheep hemoplasmosis has been historically performed by visualization of compatible morphologic structures on RBCs during blood smears examination (NEITZ, 1934; SHERIFF et al., 1966; DADDOW, 1979; MASON and STATHAM, 1991). However, this diagnostic method may present low sensitivity and specificity when compared to molecular methods. Currently, molecular techniques, such as the polymerase chain reaction (PCR) with protocols aiming to detect genetic material from *M. ovis* has being used in sheep, goats and wild ruminants worldwide (OHTAKE et al., 2011; GRAZZIOTIN et al, 2011; GRAZZIOTIN et al., 2011b; BOES et al., 2012; HAMPEL et al., 2014; JOHNSON et al, 2016; AKTAS, OZUBEK, 2017; MACHADO et al., 2017; MARTINEZ-HERNANDEZ et al., 2018).

Mycoplasma ovis has been reported in sheep herds from different countries, such as Canada (NEIMARK et al., 2004); Japan (TAGAWA et al., 2012), Turkey (AKTAS, OZUBEK, 2017), China (WANG et al., 2017) and Hungary (HORNOK et al., 2018). In South America, only few reports of M. ovis infection in sheep from Argentina (AGUIRRE et al., 2009), and goats (MACHADO et al., 2017) and deer from Brazil (GRAZZIOTIN et al., 2011; GRAZZIOTIN et al., 2011b).

Brazilian sheep herd has been estimated in almost 18 million animals. Paraná State, located in the southern region of the country, has an estimated herd of more than 500,000 sheep (IBGE, 2017). Sheep farming has been considered a growing business for Paraná producers, most because of its profitability and demand (EMATER, 2018). Co-infection of M. ovis and other pathogens, such as nematodes and tick-borne agents, may lead hosts to develop anemia and may reflect on production decay and mortality in small ruminants

(HORNOK et al., 2009; ABDULLAH et al., 2013). Although the ovine production has a notable importance, studies regarding *M. ovis* occurrence in Brazilian sheep have not been performed to date. Thus, studies aiming to elucidate the distribution of *M. ovis* in Brazilian herds of sheep are needed.

2 OBJECTIVES

2.1 General objectives

To identify the causative agent of anemia in a sheep herd from Bandeirantes County, Paraná State, southern Brazil.

2.2 Specific objectives

- To collect and identify tick species parasitizing the animals;
- To determine the packed cell volume (PCV) on sheep samples;
- To screen sheep blood samples for *Mycoplasma* sp., *Anaplasma* sp. and piroplasm species by conventional PCR assays;
- To perform parasitological analysis of feces (worm egg counting – EPG);
- To molecularly characterize the pathogens detected by sequencing and comparison with other isolates deposited in the GenBank.
- To perform phylogenetic analysis with the isolates obtained in the present study aiming to elucidate its evolutionary features.

3 CHAPTER ONE: OCCURRENCE OF HEMOPARASITES IN SHEEP

3.1 Sheep production

Sheep, together with goats, are believed to be the first domesticated animals after dogs in approximately 9,000 years B.C. (ZYGOIYANNIS, 2006). Current domestic breeds were probably descendant from the wild mouflon (*Ovis aries orientalis*), originated from Asia (McMANUS et al., 2010). Worldwide herd is estimated in more than 1 billion animals, with the greater proportions concentrated in Asia, Oceania and Africa continents. South America represents 6.8% from the world sheep production (FAO, 2016). Although sheep are raised primarily for meat purposes, these animals provide a wide range of products, such as milky, skin and wool (ZYGOIYANNIS, 2006).

Regarding to Brazil, sheep were introduced by Portuguese colonizers resulting in well adapted breeds distributed through the country (PAIVA et al., 2005). Different sheep breeds may be found in Brazil, being all originated from exotic breeds or adapted in the country. Although these breeds present a variety of aptitudes, sheep production in Brazil is mainly directed to meat (MARIANTE et al., 2003).

In 2016, the Brazilian sheep herd reached the estimated number of almost 18,000,000 of animals added to an increasing long-term scenario of production and consumption of sheep products, foreseen by Brazilian researchers (EMBRAPA, 2017; IBGE, 2017). Paraná State, located in the southern region, have an estimated herd of more than 500,000 sheep, representing 3,12% from the national herd (IBGE, 2017) and considered a growing business (EMATER, 2018), counting with financial incentives from the government (FAEP, 2017).

3.2. Hemoparasite infections in sheep

A wide range of hemoparasites, such as bacteria and protozoa, are described infecting sheep from worldwide. Molecularly detected hemoparasite from sheep hosts found in the present review are summarized in Table 1.

Table 1. Reports of hemoparasites molecularly detected in sheep

Pathogen Group	Species	Occurrence	Reference
Anaplasmataceae	*Anaplasma phagocytophilum*	China, Czech Republic, France, Italy, Norway, Saudi Arabia, Slovakia.	Ladbury et al., 2008; Zhan et al., 2010; Torina et al., 2010; Derdakova et al., 2011; Dugat et al., 2014; Yang et al., 2015; Shabana et al., 2018.
	A. phagocytophilum-like	Tunisia	Ben Said et al., 2015; Ben Said et al., 2017.
	A. ovis	Algeria, China, Hungary, Iran, Iraq, Italy, Mongolia, Portugal, Saudi Arabia, Slovakia, South Africa, Sudan, Tunisia, Turkey.	Bekker et al., 2002; Hornok et al., 2007; Torina et al., 2010; Derdakova et al., 2011; Jalali et al., 2013; Renneker et al., 2013; Yang et al., 2015; Aouadi et al., 2017; Ochirkhuu et al., 2017; Yousefi et al., 2018; Aktas, Ozubek et al., 2018; Ringo et al., 2018; Shabana et al., 2018;
	A. bovis	China, Tunisia	Ben Said et al., 2015b; Zhang et al., 2016; Belkahia et al., 2017; Wang et al., 2018; Yang et al., 2018.
	A. marginale	Iran	Jalali et al., 2013; Yousefi et al., 2017.
	A. platys-like	Tunisia	Ghai et al., 2016; Ben Said et al., 2017b
	A. capra	China	Yang et al., 2018
	Ehrlichia ruminantium	Mozambique, South Africa	Bekker et al., 2002; Ringo et al., 2018.
Piroplasmids	*Theileria lestoquardi*	Sudan, Iran, Tunisia	Heidapour Bami et al., 2009; Zaeemi et al., 2011; Rjeibi et al., 2016b; Ali et al., 2017.
	T. luwenshuni	China, Turkey, Spain	Nagore et al., 2004; Yin et al., 2007; Chen et al., 2014; Ros-Garcia et al., 2016; Bilgic et al., 2017.

T. uilenbergi	China, Iran, Turkey	Yin et al., 2007; Renneker et al., 2013; Bilgic et al., 2017.
T. ovis	Algeria, China, Ethiopia, Pakista, Saudi Arabia, South Africa, Spain, Sudan, Tunisia, Turkey	Nagore et al., 2004; Altay et a., 2005; Zaeemi et al., 2011; Li et al., 2011; Shahzad et al., 2013; Gebrekidan et al., 2014; Auoadi et al., 2017; Ringo et al., 2018; Mghirbi et al., 2018; Alazani et al., 2018;
Theileria sp. OT3	Caribbean, China, Iran, Italy, Spain, Turkey	Nagore et al., 2004; Aydin et al., 2013; Tian et al., 2014; Giansgapero et al., 2015; Ros-Garcia et al., 2016; Zhang et al., 2015; Bilgic et al., 2017; Tabaei et al., 2018.
T. separata	Ethiopia, Sudan, Turkey	Gebrekidan et al., 2014; El Imam et al., 2016; Bilgic et al., 2017.
Theileria sp. MK	Turkey	Altay et al., 2012; Aydin et al., 2013; Ozubek, Aktas et al., 2016.
T. annulata	Iran, Turkey	Zaeemi et al., 2011; Ozubek, Aktas et al., 2016.
Babesia ovis	Iran, Iraq, Israel, Pakistan, Portugal, Spain, Tunisia, Turkey.	Aydin et al., 2013; Shahzad et al., 2013; Renneker et al, 2013b; Horta et al., 2014; Ros-Garcia et al., 2016; Erster et al., 2016; Rjeibi et al., 2016; Bazmani et al., 2018;
B. motasi	China, Italy, Spain	Nagore et al., 2004; Liu et al., 2007; Giangaspero et al., 2014; Ros-Garcia et al., 2016.

	B. crassa	Turkey, Iran	Schnittger et al., 2003; Bilgic et al., 2017.
	B. crassa-like	China	Jia et al., 2018.
	B. lengau-like	Greece	Giardini et al., 2012
Hemothropic mycoplasma	*Mycoplasma ovis*	Argentina, Canada, China, Hungary, Japan, Turkey	Hornok et al., 2004; Hornok et al., 2009; Aguirre et al., 2009; Tagawa et al., 2012; Aktas, Ozubek, 2017; Wang et al., 2017

3.3 Anaplasmataceae

Since 2001, Anaplasmataceae family is reorganized in four genera comprising *Anaplasma*, *Ehrlichia*, *Cowdria* and *Neorickettsia* species (DUMLER et al., 2001). Regarding this family, *Anaplasma phagocytophilum* and *Anaplasma ovis* are frequently described as disease causing in sheep (TORINA et al., 2010; RENNEKER et al., 2013). Sheep presenting poor health conditions are reported as more susceptible to be infected by multiple *Anaplasma* species (TORINA et al., 2010).

In 1932, a "tick-borne fever" (TBF) was described in sheep herds from the United Kingdom, causing febrile phases and loss of body weight in the animals. The infective agent was present in blood and spleen and its transmission was associated with *Ixodes ricinus* ticks (GORDON et al., 1932). The species *A. phagocytophilum*, a gram-negative obligatory bacterium, is responsible for infect granulocytes from a wide range of mammals and considered the causative agent of TBF in domestic ruminants (LALOY et al., 2009; RIKIHISA, 2011). Sheep may act as reservoirs for this pathogen in some areas, harboring persistent infections (THOMAS et al., 2012). These animals may play an important role in the epidemiological chain of *A. phagocytophilum*, being an infection source for susceptible ticks (OGDEN et al., 2002). Moreover, different genotypes may occur in the same flock (LADBURY et al., 2008; DUGAT et al., 2017) and wild animals may be involved in the epidemiological chain (ZHAN et al., 2010). Recently, intrauterine infection was also related to *A. phagocytophilum* persistently infected sheep (STUEN et al., 2018).

Although its occurrence in small ruminants is concentrated in European countries (LADBURY et al. 2008; WOLDEHIWET, 2010; STUEN, 2016), reports of *A. phagocytophilum* and *A. phagocytophilum*-like strains in sheep may be found in Asia (ZHAN et al., 2010; YANG et al., 2018), Middle East (SHABANA et al., 2018) and Africa (BEN SAID et al., 2015; BEN SAID et al., 2017). In Brazil, although some reports indicate genetically similar strains infecting wild animals (MONGRUEL et al., 2017), *A. phagocytophilum* occurrence was not effectively recognized.

Anaplasma ovis is a more selective pathogen, infecting mainly sheep and goats (KUTTLER, 1984) and it is considered a neglected infection of small ruminants that may lead to economic losses (RENNEKER et al., 2013). This pathogen infects erythrocytes and is often related to cause mild pathogenicity in sheep (HORNOK et al., 2007; RENNEKER et al., 2013). Nevertheless, cases of anemia may occur (YASINI et al., 2012) and animal condition may be aggravated when co-infection with other hemoparasites occur (AKTAS, OZUBEK, 2018). DNA from this pathogen have already been detected in milk from healthy sheep in China (ZHANG et al., 2016b). *Dermacentor andersoni* and *Rhipicephalus bursa* ticks are associated with transmission (KOCAN et al. 1991; DE LA FUENTE et al., 2005). *Rhipicephalus turanicus* tick may be also related to infections in sheep (AKTAS, OZUBEK, 2018).

Occurrence of *A. ovis* infecting sheep herds is reported from a different range of areas from the world, such as Algeria (AOUADI et al., 2017), Italy (DE LA FUENTE et al., 2005), Hungary (HORNOK et al., 2007), Iran (JALALI et al., 2013), Niger (DAHMANI et al., 2017), Turkey (AKTAS, OZUBEK, 2018) and China (YANG et al., 2015; ZHANG et al., 2016; YANG et al., 2018). In the United States, this pathogen is reported infecting wild ruminants (GOFF et al., 1993; DE LA FUENTE et al., 2006). *Anaplasma ovis* is considered highly involved in concurrent infections with other hemoparasites in sheep (JALALI et al., 2013; YANG et al., 2015; SEVINC et al., 2018; RINGO et al., 2018) and its occurrence is reported during all year seasons (BELKHIA et al., 2017). To date, there is no report of *A. ovis* occurring in any animal species in Brazil.

Other *Anaplasma* species are described infecting sheep around the world, as *Anaplasma capra,* a recently proposed new species that may also affect goats and humans in China (LI et al., 2015; YANG et al., 2018). Further investigations about this pathogen still needed. Anaplasma marginale, well recognized as one of the most important pathogens for cattle, occurs mostly in subtropical and tropical regions and infects cattle being transmitted by

Rhipicephalus microplus ticks (KOCAN et al., 2010). This pathogen was reported infecting sheep from Iran (JALALI et al., 2013l; YOUSEFI et al., 2017) and, although there is no report of sheep infection in Brazil, goats were recently described being infected by *A. marginale* in the country (DA SILVA et al., 2018).

Strains related to *Anaplasma platys* were detected infecting sheep from Tunisia (GHAI et al., 2016; BEN SAID et al., 2017). This pathogen is known to infect platelets from dogs (DUMLER et al., 2001), although there are evidences of genetically close strains occurring in different host species, such as cats and buffaloes (ZOBBA et sl., 2015; MACHADO et al., 2016). Infection by *A. platys*-like strains in small ruminants need further investigations. *Anaplasma bovis* is a monocyte parasite that mainly affects cattle, causing fever, anorexia, lymph node enlargement and mucosal pallor (MELO JUNIOR et al., 2010). However, its occurrence in sheep is frequently reported in China and Tunisia (BEN SAID et al., 2015b; ZHANG et al., 2016; BELKHIA et al., 2017; WANG et al., 2018; YANG et al., 2018.). In some studies, *A. bovis* prevalence in ovine showed to be even greater than *A. phagocytophilum* (ZHANG et al., 2016). A study conducted in Tunisia reported that *A. bovis* infection may be frequently mixed with *A. ovis* in small ruminants (BELKHIA et al., 2017).

Ehrlichia ruminantium, a gram-negative bacterium that replicates in endothelial cells, is considered the causative agent of heartwater. This pathogen affects domestic and wild ruminants mainly in the sub-Saharan Africa region (DUMLER et al., 2001; ALLSOPP et al., 2010). The disease name is given to pericardial effusions that may develop in small ruminants (ROBSON, ROBSON, 2016). Economic losses caused by the disease are considered expressive in affected areas (MELTEZER et al., 1996) and infections may lead mortalities rates above 90% in sheep (NEITZ et al., 1964). Its transmission is related to ticks from genus *Amblyomma* (BEKKER et al., 2002; ALLSOPP et al., 2010), but it was also found infecting *Rhipicephalus microplus* ticks (BIGUEZOTON et al., 2016). Mixed infections involving other blood parasites such as *A. ovis* and *Theileria ovis* may occur in sheep (RINGO et al., 2018). Although there are no reports about this agent infecting livestock in Brazil, a closely genotype of *Ehrlichia* sp. was found infecting a horse from the southern part of the country (VIEIRA et al., 2018).

3.4 Piroplasmida

Piroplasmids are protozoa agents belonging to order Piroplasmida. Regarding to ovine,

Babesia spp. and *Theileria* spp. represent the most important genera from this group with various species. These pathogens were considered neglected when compared to cattle piroplasmids. However, studies about small ruminants piroplasmids have increased together with the economic interest in these animals (SCHNITTGER et al., 2003).

Regarding ovine theileriosis, clinical signs in sheep may be presented as fever, cough, anorexia, mucosal pallor and ruminal hypomotility (HASSAN et al., 2015) and different species may act as the causative agent. Theileriosis transmissions in sheep are related to ticks from genera *Hyalomma* and *Rhipicephalus* (MORZARIA, 1998).

The species *Theileria lestoquardi*, considered the causative of the malignant theileriosis disease, is appointed as a highly pathogenic species, reaching morbidity and mortality rates of 100% and 90% in sheep, respectively (HOOSHMAND-RAD, HAWA, 1973). Its occurrence has been reported in Sudan, Iran and Tunisia (HEIDAPOUR-BAMI et al., 2009; ZAEEMI et al., 2011; RJEIBI et al., 2016b; ALI et al., 2017).

Phylogenetic analysis of *Theileria* sp. China 1 and *Theileria* sp. China 2 genotype revealed that these species genetically differ from *T. lestoquardi* and may present a pathogenic course in sheep. Moreover, a new classification of these species was proposed, being named as *Theileria luwenshuni* and *Theileria uilenbergi*, respectively (YIN et al., 2007). Once *Theileria* sp. OT1 genotype presented high genetical similarity with *Theileria* sp. China 1 it was also proposed as *T. luwenshuni* (ROS-GARCIA et al., 2013). Althoug *T. luwenshun*i and *T. uilenberg* were believed to circulate only in China, reports from other regions such as Spain (NAGORE et al., 2004), Iran (RENNEKER et al., 2013) and Turkey (BILGIC et al., 2017).

Some *Theileria* species appears to be less pathogenic in immunocompetent animals, such as *Theileria ovis* (ALANI, HEBERT, 1988; LI et al., 2011). Besides that, this species is frequently reported in different regions of the world, such as Spain (NAGORE et al., 2004), China (LI et al., 2011), Algeria (AOUADI et al., 2017) and South Africa (RINGO et al., 2018). Also, strain *Theileria* sp. 0T3 have been isolated in sheep from Spain (NAGORE et al., 2004), Turkey (AYDIN et al., 2013), China (TIAN et al., 2014), Italy (GIANGASPERO et al., 2015), Caribbean Islands (ZHAG et al., 2015) and Iran (TABAEI et al., 2018). Although its widely occurrence, pathogenicity related to this strain is not fully elucidated but abortions may be associated (NAGORE et al., 2004). Tick species *R. sanguineus*, *Haemaphysalis puntacta* and *Hyalomma detritum* are related as vectors (TIAN et al., 2014).

Species such as *Theileria separata*, *Theileria* sp. MK and *Theileria annulata* are scarcely reported in sheep by molecular techniques. *Theileria separata* is reported in sheep from Ethiopia (GEBREKIDAN et al., 2014), Sudan (EL IMAM et al., 2016) and Turkey (BILGIC et al., 2017), while *Theileria* sp. MK reports appears to be concentrated only in Turkey (ALTAY et al., 2012; AYDIN et al., 2013; OZUBEK, AKTAS et al., 2016). Although *T. annulata* is well recognized as a blood parasite from bovine hosts, it was reported occurring in sheep from Iran and Turkey (ZAEEMI et al., 2011; OZUBEK, AKTAS et al., 2016). Pathogenicity and vectors involved still need further investigations.

Although occurrence of *Theileria* species is described to occur in tropical and sub-tropical countries (HOOSHMAND-RAD, HAWA, 1973), reports appears to be concentrated in Africa (RJEIBI et al., 2016; ALI et al., 2017; RINGO et al., 2018), Asia (JIANXUN, HONG, 1997; ALTAY et al., 2005; YIN et al., 2007; AYDIN et al., 2013), Middle East (TAGELDIN et al., 2005; ZAEEMI et al., 2011; HASHEMINISAB et al., 2018) and some Europe regions (ALANI, HEBERT, 1988; GIANGASPERO et al., 2015). In Brazil, descriptions of *Theileria* detected in horses, buffaloes and wild animals (SILVEIRA et al., 2016; FERREIRA et al., 2016; SILVEIRA et al., 2017; VIEIRA et al., 2018b) were performed, but to date there are no reports about ovine theileriosis in the country.

Babesiosis is also caused by more than one species of *Babesia* spp. in sheep. Fever, haemoglobinuria and anemia are described as babesiosis clinical manifestations in ovine (YERUHAM, 1998). The disease is considered more life-threatening to sheep when compared to theileriosis and anaplasmosis (SEVINC et al., 2018). The species *Babesia ovis* and Babesia motasi are considered highly pathogenic for small ruminants (HAGHI et al., 2013) and transmitted by *Rhipicephalus* and *Haemaphysalis* ticks, respectively (UILENBERG, 2006). Besides that, vector species and pathogenicity of *Babesia crassa* still need further investigations (UILENBERG, 2006).

Species *B. ovis* is a wide distributed in Middle East, Europe and Asia (AYDIN et al., 2013; SHAHZAD et al., 2013; RENNEKER et al, 2013b; HORTA et al., 2014; ROS-GARCIA et al., 2013; ERSTER et al., 2016; RJEIBI et al., 2016; BAZMANI et al., 2018), meanwhile *B. motasi* and *B. crassa* are less reported using molecular tools. *Babesia crassa* is more related to reports from Asia and Middle East (SCHNITTGER et al., 2003; BILGIC et al., 2017) and *B. motasi* is described occurring in Asia and Europe (NAGORE et al., 2004; LIU et al., 2007; GIANGASPERO et al., 2014; ROS-GARCIA et al., 2013).

Furthermore, *Babesia* genotypes that remain not fully characterized are reported. A

genotype closely related to *Babesia lengau*, a novel species found in African cheetah (*Acinonyx jubatus*), are described infecting sheep from Greece that were presenting icteric membranes, anemia and slight leukocytosis (GIARDINI et al., 2012). *Babesia crassa*-like agents were identified in humans with clinical symptomatology in China, raising concerns about zoonotical transmission (JIA et al., 2018). In Brazil, although *Babesia* species are well documented in different animal hosts, there are no reports involving sheep until today.

3.5 Hemotropic mycoplasmas

The genus *Mycoplasma* is comprised within the Mollicutes class, and are distinguished by cell wall lackness (TRATCHENBERG, 2005). Species from *Haemobartonella* and *Eperythrozoon* genera were re-classified into the genus Mycoplasma after studies elucidating its real phylogenetic relationship (RIKIHISA et al., 1997; NEIMARK et al., 2001). Thus, classification of some *Mycoplasma* species as hemotropic mycoplasmas (hemoplasmas) were performed. These species, also known as hemoplasmas, are small and pleomorphic bacteria of red blood cells that may infect many different species of vertebrate hosts (MESSICK et al., 2004).

Since 2004, the recognized pathogen *Eperythrozoon ovis* has been described as *Mycoplasma ovis*. This species is described as a wall-less bacterium that infect sheep and goat erythrocytes (NEIMARK et al., 2004). Weight loss (MARTINEZ- HERNANDEZ et al., 2018), hyperthermia, mucosal pallor and hemolytic anemia (NEIMARK et al., 2004; AKTAS, OZUBEK, 2017) were clinical manifestations linked to *M. ovis* infection in ovine. Concurrent infections with internal parasites may increase the pathogenicity of *M. ovis* in sheep (OHTAKE et al., 2011).

Descriptions of *M. ovis* occurrence in sheep herds from different places of the world, such as Japan (TAGAWA et al., 2012), Turkey (AKTAS, OZUBEK, 2017), China (WANG et al., 2017) and Hungary (HORNOK et al., 2018) may be found. However, only few reports are originated from South America, being concentrated in Argentina (AGUIRRE et al., 2009). In Brazil, *M. ovis* has been reported in deer (GRAZZIOTIN et al., 2011; GRAZZIOTIN et al., 2011b) and goats (MACHADO et al., 2017). Sheep located in Japan that were originally from Australia were infected my hemotropic mycoplasmas (TAGAWA et al., 2012), raising a concern about sheep trade and pathogen distribution worldwide. Moreover, a *M. ovis*-like infecting a veterinarian has been found (SYKES et al., 2010), which

highlights for the zoonotical potential of this bacterium. Transmission route or the putative vector remains to be investigated.

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5 CHAPTER TWO: MANUSCRIPT FOR SUBMISSION

Title: Haplotype diversity of Mycoplasma ovis in sheep from Paraná state, southern Brazil.

5.1 Abstract

Mycoplasma ovis is a wall-less bacterium that infects sheep and goat erythrocytes. Infection by *M. ovis* in sheep may leads to weight loss, submandibular edema, hyperthermia, mucosal pallor and hemolytic anemia. Although the importance of sheep industry in Brazil, no studies regarding *M. ovis* infection have been reported in the country. Moreover, *Anaplasma marginale* has been detected in goats from Brazil. Accordingly, the aims of this study were to i) screen sheep blood samples for *Mycoplasma* sp. and the tick-borne agents *A. marginale* and piroplasms infection by PCR; ii) identify the tick species parasitizing the animals; iii) evaluate the presence of co-infection with gastrointestinal (GI) nematodes by McMaster Fecal Egg Counting (FEC); and iv) determine the association between the presence of anemia and infection by these agents in a sheep herd from Bandeirantes City, Paraná State, southern Brazil. Seven out of 42 (16.6%; 95% CI: 8.32–30.6%) sheep were positive for *Mycoplasma* spp. and all tested negative for *A. marginale* and piroplasms by PCR. Two animals were infested by ticks. FEC was performed in 38 sheep and 24 (63,15%; 95% CI: 47.2 – 76.6%) presented > 500 eggs per gram (EPG). Nine out of 42 (21,42%; 95%CI: 11.71-35.94%) animals were anemic. Sequencing of three *Mycoplasma*-positive sample showed ≥ 99% identity to multiple M. ovis 16S rDNA gene sequences deposited in GenBank. Obtained sequences clustered together with *Mycoplasma* sp. and *Mycoplasma ovis* through Bayesian inference. Haplotype analysis showed three different genotypes. *Mycoplasma* sp. occurs in sheep from the north region of Paraná State and may develop anemia in co-infected animals.

Key-words: Small ruminants, hemoplasmas, *Mycoplasma ovis*, *Anaplasma marginale*, piroplasms.

5.2. Introduction

Brazilian sheep herd has been estimated in more than 18 million animals. The southern region of the country concentrates 30% of the Brazilian herd, which are mainly focused on

meat production (IBGE, 2016). The gastrointestinal (GI) parasite *Haemonchus contortus* is responsible for great impact to the sheep industry (Lane et al., 2015), since it infects the sheep abomasum, causing an acute hemorrhagic anemia crisis which may leads to sudden death (Taylor et al., 2007). In sheep, although anemia is commonly linked to *H. contortus* infection, it is important to state that other infectious agents, such as hemotropic mycoplasmas (hemoplasmas) and tick-borne diseases (TBDs) agents, may also leads to anemic crisis (Yeruham et al., 1998; Neimark et al., 2004; Hornok et al., 2009; Alessandra and Santo, 2012), and historically have not been included as differential diagnosis of anemia by veterinary practitioners in Brazil.

Hemoplasmas are small and pleomorphic bacteria of red blood cells that may infect many different species of vertebrate hosts (Messick et al., 2004). In small ruminants, two hemoplasma species have been initially found *Mycoplasma ovis* (formerly *Eperythrozoon ovis*) and '*Candidatus* Mycoplasma haemovis' (Hornok et al., 2012). However, the complete genome sequence of *M. ovis* strain Michigan revealed two copies of the 16S rDNA genes, which corresponded to the previously reported sequences for *M. ovis* and '*Ca*. M. haemovis' (Deshuillers et al., 2014). In sheep, *M. ovis* infection may cause weight loss, hyperthermia, mucosal pallor and hemolytic anemia (Neimark et al., 2004; Aktas and Ozubek, 2017; Martinez-Hernandez et al., 2019). In Brazil, *M. ovis* has been detected in deer (Grazziotin et al., 2011a;2011b) and goats (Machado et al., 2017).

Rhipicephalus microplus ticks, are endemic in Brazil (Dantas-Torres et al., 2009). Despite host specificity of *R. microplus* for cattle (Ma et al., 2016), this tick species may be found parasitizing small ruminants (Brito et al., 2005), and thus, may transmit pathogens. In Brazil, *Anaplasma marginale*, the causative agent of bovine anaplasmosis, is transmitted by *R. microplus* ticks and this bacterium has been detected in goats from the northeastern region of the country (Da Silva et al., 2018). Additionally, this bacterium has been detected in sheep from Iran (Yousefi et al., 2017). However, the epidemiological and clinical impact of *A. marginale* infection in small ruminants remains to be fully established.

A wide range of piroplasm species, mainly from the genus *Theileria* and *Babesia*, may infect sheep. Although piroplasmid infections in small ruminants may be considered neglected when compared to cattle infections, studies have increased together with the economic interest in these animals (Schnittger et al., 2003). These infections may cause fever, anorexia, mucosal pallor, hemoglobinuria and anemia (Yeruham et al., 1998; Hassan et al., 2015). *Rhipicephalus*, *Hyalomma* and *Haemaphysalis* ticks are related as putative vectors (Morzaria, 1998; Tian et

al., 2004; Uilenberg, 2006). In Brazil, studies on piroplasmid infection in sheep have not been reported.

Co-infection between *M. ovis* and other pathogens, such as nematodes and TBDs agents, may leads to hemolytic anemia and reflect on production decay and mortality (Hornok et al., 2009; Abdullah et al., 2013). Although the ovine production has a notable importance, no studies on *M. ovis*, *A. marginale* and piroplasms occurrence in sheep from Brazil have been reported. Accordingly, the aims of this study were to i) screen a sheep herd for *Mycoplasma* sp. and the TBDs agents *A. marginale* and piroplasms by PCR; ii) identify the tick species parasitizing the animals; iii) evaluate the presence of co-infection with gastrointestinal (GI) nematodes by McMaster Fecal Egg Counting (FEC); and iv) determine the association between the presence of anemia and infection by these agents in a sheep herd from Bandeirantes City, Paraná State, southern Brazil.

5.3 Material and Methods

5.3.1 Ethical approval

This study was approved by the Ethics Committee for Animal Experimentation and Animal Welfare at Universidade Federal do Paraná (protocol nº 030/2019) and conducted according to the ethical principles of animal experimentation, adopted by the Brazilian College of Animal Experimentation.

5.3.2 Study

A total of 42 sheep from Bandeirantes County (23°06'28"S 50°21'36"W), Paraná State, southern Brazil, were evaluated for the presence of hemoplasmas, *Anaplasma* spp., piroplasms and GI parasites infection. Animals were co-grazed with cattle in a paddock, where tick infestation is common during all year.

5.3.3 Sampling

Sheep blood samples (up to 5 mL) were collected by venipuncture of the jugular vein using commercial tubes containing EDTA (BD Vacutainer®, Franklin Lakes, NJ, USA). Fecal samples were obtained directly by rectal collection, identified and stored in isothermal recipients until analysis.

Ticks found on animals were directly removed using a commercial hook (O'TOM Tick Twister®, Lavancia, France), and kept in absolute ethanol-labeled tubes until identification according to morphological taxonomic keys (Aragão and Fonseca, 1961; Guimarães et al., 2001).

5.3.4 Packed cell volume evaluation

The packed cell volume (PCV) was measured by routine centrifugation. A PCV value of < 0.27 L/L was used as an indicator of anemia (Weiss et al., 2010). Thereafter, blood aliquots were stored at −20 °C until molecular testing.

5.3.5 Parasitological analysis

Fecal samples were analyzed by the eggs per gram (EPG) technique (Gordon & Whitlock 1939). Animals presenting EPG > 500 were considered positive (Ueno and Gonçalves, 1998).

5.3.6 DNA extraction

Isolation of genomic DNA from sheep blood samples was performed using a commercial kit (llustra™ blood genomicPrep Mini Spin Kit, GE Healthcare, Little Chalfont, UK). Nuclease-free water was used in parallel as negative control to monitor cross-contamination.

5.3.7. Polymerase chain reactions (PCR)

DNA samples were submitted to the gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene PCR, as previously described (Birkenheuer et al., 2003). GAPDH-positive samples were screened for *Mycoplasma* spp. by a PCR targeting a fragment

(900 bp) of the 16S rRNA gene of this bacterium (Hoelzle et al., 2011; Machado et al., 2017). Additionally, samples were also tested by PCR assays targeting a fragment (870 bp) of msp4 gene of *A. ovis*/*A. marginale* (De La Fuente et al., 2007), and a fragment (551 bp) of the 18S rDNA gene of piroplasms (Almeida et al., 2012)

5.3.8 Sequencing

Amplicons obtained from four Mycoplasma-positive samples were purified by enzymatic purification (ExoSAP-IT™ PCR Product Cleanup Reagent, Thermo Scientific, Waltham, USA), evaluated by spectrophotometry for concentration and purity (NanoDrop™ One Spectrophotometer, Thermo Scientific, Waltham, USA), and sequenced in both directions by the Sanger method (3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Thereafter, sequences were subjected to BLASTn analysis (Altschul et al., 1990) for determining the identity with the sequences deposited in the GenBank database. The nucleotide sequences of the *M. ovis* amplified herein were submitted to the GenBank® database (Genbank® accession numbers. XXX, XXX and XXX).

5.3.9 Phylogenetic analysis

Sequences were aligned using MAFFT 7.110 (Katoh and Standley, 2013). Best-fit evolutionary model was estimated as F81+I+G using jModeltest 2.1.4 (Darriba et al. 2012). The Bayesian Information Criterion (BIC) algorithm was used for phylogenetic inference. Three reconstruction was made with Fig.2 Tree 1.4.2. Polymorphisms were investigated in isolated sequences using DnaSP software v. 6.12.03.

5.4 Results

The mean PCV concentration for sheep was 0.30 L/L. Nine out of 42 (21.42%; 95% CI: 11.71-35.94%) animals were anemic. A total of 38 sheep were evaluated by EPG. Four out of 42 animals did not present feces at the time of sampling and were not evaluated. Twenty-four (63.15%; 95% CI: 47.2 – 76.6%) animals presented EPG values >500 and were considered positive for Strongylida type-eggs. A total of 24 adult tick specimens were collected from two/42 (4.76%; 95% CI: 1.32-1.57%) animals and all were identified as *R. microplus* species.

All samples consistently amplified the sheep GAPDH gene. Seven out of 42 (16.6%; 95% CI: 8.32–30.6%) animals were PCR-positive for *Mycoplasma* spp. Only one *Mycoplasma* spp. PCR-positive animal was anemic (PCV = 0.17L/L). The PCR results, parasitological analysis and presence of ticks has been summarized in Table 2. All samples tested negative to *Anaplasma* spp. and piroplasm species by PCR.

All *Mycoplasma*-positive samples sequenced showed ≥99% identity with multiple M. ovis 16S rDNA gene sequences deposited in GenBank (accession nos. KU98374, KU983745, KU512718). The phylogeny showed two great clades dividing *Mycoplasma haemofelis* and *Mycoplasma suis* groups. Our sequences clustered together in a clade including *M. ovis* and *Mycoplasma* sp. sequences, inside the *M. suis* group. Polymorphism analysis revealed three different haplotypes from isolated sequences.

5.5 Discussion

Anemia is considered a concerning condition for animal health and production. Clinical manifestations may be represented by weakness, pallor of mucous membranes, hemoglobinuria and icterus. Regarding laboratorial findings, values of packed cell volume, total erythrocyte count and hemoglobin concentrations should be reduced (WEISS et al., 2010). In sheep, causatives of anemia are commonly linked to parasitological causes.

Herein we report the occurrence of *M. ovis* in sheep from Paraná State. Although there are no studies involving *M. ovis* occurrence in sheep from Brazil, our prevalence of 16.6% of infected animals is low when compared with data regarding *M. ovis* infection in different host species from Paraná State and Brazil. A study reporting *Mycoplasma* sp. occurrence in goats from northeastern region obtained prevalence of 39.30% (Machado et al., 2017). Regarding to wild animals, an overall prevalence of 58% of M. ovis infected deer from Brazilian southeast and midwestern regions is reported (Grazziotin et al., 2011a). In Paraná State, captive cervids have reached a prevalence of 87% of infection by *M. ovis* in the municipality of Foz do Iguaçu (Grazziotin et al., 2011b).

Infection by *M. ovis* in sheep may produce mild to severe anemia (Neimark et al., 2004). Our findings show that infection by *Mycoplasma* sp. in ovine may be often conducted as subclinical conditions, once only one positive animal presented anemia. Recently, a study conducted in Turkey showed that prevalence of hemotropic mycoplasma infection is lower in sheep with clinical signs than that classified as “healthy” (Aktas and Ozubek, 2017).

In the present work, we also evaluated the occurrence of GI parasites in sampled sheep. Concurrent infections with internal parasites may increase the pathogenicity of *M. ovis* in sheep (Ohtake et al., 2011). Infections by GI parasites and *M. ovis* in goats may lead animals to present anemia, pale mucous membrane and pasty feces (Abdullah et al., 2013). The only animal from our study that presented positive results for hemoplasmas and anemia obtained a low value of PCV (0.17 L/L). This animal has also obtained high values of EPG (1.700) from eggs identified as Strongylida type. The Strongylida suborder is known to shelter important parasites for ovine production, such as *Haemonchus contortus*, that may lead animals to severe anemic conditions (Amarante et al., 2014). Probably, the co-infection of GI parasites and hemoplasmas have worsened the animal health condition, which may represent an alert about hemoplasmas diagnosis in ovine herds with nematode infections occurrence.

The mean value of PCV obtained by *Mycoplasma* sp. infected sheep from the present study was 0.28 L/L, being within the reference values for this animal species (Weiss et al., 2010). Similar results were obtained in a study conducted in Argentina, where infected animals have mean value of PCV considered inside the normal range (Aguirre et al., 2009). Although sheep from our study were also co-infected with GI parasites, and clinical evaluation was unfortunately not performed, these results may suggest that these animals were well supporting the infections in relation to anemia development. Considering that eight hemoplasma negative animals obtained low PCV results, these results may be linked to other causes as other internal parasites or even hemoparasites do not target in this study.

Anaplasma marginale is a common tick-borne pathogen that infects primarily cattle from tropical and subtropical areas (Kocan et al., 2014). Recently, this bacterium was detected infecting sheep from Iran (Yousefi et al., 2017). Although there are no reports about *A. marginale* infection in sheep from Brazil, goats kept in a co-grazing system with cattle were found infected in the Northeastern region of the country (Da Silva et al., 2018). Although the main species vector involved in *A. marginale* transmission - *R. microplus* ticks - was found infecting animals from our study, all animals sampled presented negative results for Anaplasma spp. These results may suggest that acute infections caused by Anaplasma agents did not occur in the studied herd in the sampling moment.

The phylogenetic analysis showed that our sequences formed a cluster together with M. ovis sequences from different hosts and geographical origins. Sequences 3 and 5 from the present study clustered together with *M. ovis* isolated from japanese serows (*Capricornis crispus*) from Japan (AB571119), meanwhile sequence 7 clustered together with *M. ovis*

isolated in sheep from Turkey (MF377458). All *M. ovis* sequences and *M. ovis*-related sequences formed a great cluster together within the *Mycoplasma suis* group.

Although our phylogenetic tree mostly presented satisfactory probabilities values, values regarding the study sequences presented low percentages. Polymorphism analysis showed three different haplotypes from the three sequences isolated. The presence of polymorphisms may cause incongruities in post probabilities values, although closely positions were obtained. Meanwhile, our phylogenetic results showed marked differences from sequences isolated in a same herd. Genotype diversity of *M. ovis* are also reported in flocks from China (Wang et al., 2017) and Mexico (Martinez-Hernandez et al., 2019).

The 16S rDNA gene is widely used for phylogenetic analysis, however characteristics as intra-genomic heterogenicities are considered a limiting factor (Rejendhran and Gunesakaran et al., 2011). In 2014, genome sequencing of *M. ovis* strain Michigan was reported showing the presence of two copies of the 16S rDNA gene (Deshuillers et al., 2014). Primers used in the present study are not capable of specifically targeting one or another copy from the *M. ovis*-16S rDNA which may represent unreliable data on sequence analysis.

Regarding the tick collection, one *R. microplus*-infested animal was also considered positive for Mycoplasma sp. Tick species *Rhipicephalus bursa* was implicated as a vector of *M. ovis* in small ruminants (Neimark et al., 2004). However, studies did not find significant association between tick presence and infection by *Mycoplasma* sp. in hosts (Rjeibi et al., 2015; Machado et al., 2017). The main vector of *Mycoplasma* sp. in sheep from Paraná remains unknow.

5.6 Conclusion

Anemia causes in the studied herd may be probably linked to GI infections. Different haplotypes of *Mycoplasma ovis* occurs in sheep from the north region of Paraná State.

5.7 References

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Figure 1. Phylogenetic tree based on partial sequences of the 16S rDNA gene, showing the relationship between the Mycoplasma sp. detected in sheep from this study and other hemoplasmas by Bayesian Inference. Bacilus subitilis and Mycoplasma bovis were used as outgroups. Analyses were carried out applying the F81+I+G model and 1000 bootstrap replicates for all analyses.

Table 2. Obtained results regarding PCV values, amplification of the Mycoplasma spp. 16S rRNA fragment by PCR, EPG values and tick presence/identification in sheep from the present study.

Animal identification	PCV value	Mycoplasma PCR result	Parasitological analysis	Presence of R. microplus ticks
1	29,9	+	7.950 EPG	
2	32,3	+	250 EPG	
3	**23,9**		Negative	
4	28,1		450 EPG	
5	27,0		300 EPG	
6	31,6	+	450 EPG	
7	27,4	+	Negative	1 male and 10 females
8	**21,5**		Not collected	
9	32,4		Negative	
10	29,1		1.750 EPG	
11	32,7		Negative	
12	28,6		Negative	
13	**21,9**		5.200 EPG	
14	31,6		900 EPG	
15	**19,3**		2.750 OPG	
16	30,5		50 EPG	
17	34,4		1.550 EPG	
18	**23,6**		3.400 EPG	
19	34,4		350 EPG	
20	34,3		Not collected	
21	33,7	+	2.000 EPG	
22	**26,9**		3.500 EPG	
23	29,1	+	Not collected	
24	29,2		400 EPG	
25	38,1		150 EPG	
26	**20,9**		800 EPG	1 male and 12 females
27	29,8		1.450 EPG	
28	31,5		950 EPG	
29	29,8		2.150 EPG	
30	31,6		500 EPG	
31	28,9		3.400 EPG	
32	35,8		1.200 EPG	
33	33,1		2.150 EPG	
34	**15,0**		Not collected	
35	34,0		Not collected	
36	33,5		500 EPG	
37	32,1		2.950 EPG	
38	27,3		4.500 EPG	
39	28,2		7.000 EPG	
40	**17,6**	+	4.550 EPG	
41	29,5		1.050 EPG	
42	36,1		1.700 EPG	

6 APPENDIX: SECOND MANUSCRIPT FOR SUBMISSION

A parallel research made during the master's course.

Title: Hemotrophic hemoplasma infection in Rhesus Monkeys (*Macaca mulatta*) from a research colony in Rio De Janeiro, Brazil.

6.1 Introduction

The Mollicutes class involves about of 200 classified species of bacteria. Among them, the ones comprised in the *Mycoplasma* genus are distinguished by cell wall lack (Razin et al., 1998; Tratchenberg, 2005). However, this characteristic did not prevent *Mycoplasma* to infect a wide range of host species including mammals, birds, arthropods and plants. With a notable small genotype, these species become desirable models for genomics studies (Dybvig and Voelker, 1996).

Since few decades ago, species belonging to *Haemobartonella* and *Eperythrozoon* genus, from Anaplasmataceae family, were proposed to be re-classified into the *Mycoplasma* genus in order to reflect its phylogenetic relationship obtained by studies with the 16S rRNA gene from these species (Rikihisa et al., 1997; Neimark et al., 2001).

Such studies allowed the classification of some *Mycoplasma* species as hemotrophic *Mycoplasmas*, also known as hemoplasmas. These species are considered small pleomorphic bacteria that parasitize the surface of red blood cells from different vertebrate hosts and may lead them to anemia conditions (Messick et al., 2004). Hemoplasma infection is widely studied in a large range of animal hosts, both domestic and wild species.

The use of animal experimentation in biomedical researches had vastly increased the scientific knowledge and human health (Institute of Medicine and Nacional Research Council, 1998). Health monitoring programs in laboratory kept animals are essential once only animals free of pathogens that may modify its physiological parameters are considered suitable for research uses (Baker, 1998).

Although mucous *Mycoplasma* infections are well documented in laboratory animals, few studies are related to hemoplasmas. In research sheep, hemoplasmas caused subclinical and acute infections, raising concerns about the suitability of these animals for biomedical studies (Hampel et al., 2014). *Mycoplasma coccoides* (formerly *Eperythrozoon coccoides*) is

considered a common species that may cause latent infection in laboratory rodents (Glasgow et al., 1974). This species is reported as a cause of influence in late studies involving other hemoparasites infections such as *Plasmodium chabaudi* (Ott et al., 1967), *Trypanossoma brucei* (Molyneux, 1970) and *Plasmodium berhgi* (Andrade Jr et al., 1986). Morevorer, *M. coccoides* showed to interfere in different study fields, as host interferon production against viral infections (Glasgow et al, 1971) and significance factor in tumor studies (Nelson, 1955).

Mycoplasma muris (formerly *Haemobartonella muris*) is also well documented as a causative of latent infection in laboratory mouse (Bartlett and Pease, 1974). This species may cause synergism mechanisms in concurrent infections with *P. berghi* causing activation of latent *M. muris* with intense infection rates (Hsu and Geiman, 1952).

Regarding non-human primates (NHP), it is estimated that 100,000 to 200,000 animals are annually used in research worldwide (Carlsson et al., 2004). These animals are described as hosts of three hemoplasmas species that remain partial characterized: '*Candidatus* Mycoplasma kahanei' (Neimark et al., 2002), '*Candidatus* Mycoplasma aoti' (Barker et al., 2011) and '*Candidatus* Mycoplasma haemomacaque' (Maggi et al., 2013).

The present study aims to report *Mycoplasma* sp. infection detected in blood samples from rhesus monkey (*Macaca mulatta*) from a research center in Brazil.

6.2 Material and Methods

6.2.1 Sampling

In 2018, blood samples from rhesus monkeys kept in captivity for laboratory uses were collect twice by venipuncture and stored in tubes containing EDTA. The laboratory colony is maintained at a certified Research Center in Rio de Janeiro City, southeastern Brazil for use on studies focusing on arboviruses as Yellow Fever and Zyka.

The first collection was performed in February and a total of eight animals were sampled. Second collection were performed in the same animals in December and only six animals were sampled once two of them died.

Animals did not present ectoparasites at the time of sampling. Researchers stated that all non-human primates receive a preventive application of ivermectin monthly.

6.2.2 DNA extraction and polymerase chain reaction (PCR) assays

DNA was obtained from 200 uL of blood from each sample using a commercial kit (llustraTM blood GenomicPrep Mini Spin Kit, GE Healthcare, Little Chalfont, UK). A conventional PCR for the non-human primate house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed in all samples to ensure successful DNA extraction (Birkenheuer et al., 2003). Samples were screened for hemoplasmas using previously described pan-hemoplasma primers targeting the 16S rDNA gene (~900 bp) (Hoelze et al., 2001; Machado et al., 2017). A cat-positive blood sample for '*Candidatus* M. haemominutum' and ultrapure water were used as positive and negative controls, respectively.

6.2.3 Sequencing and phylogenetic analysis

Amplicons obtained from three samples were purified by enzymatic purification (ExoSAP-IT™ PCR Product Cleanup Reagent, Thermo Scientific, Waltham, USA), evaluated by spectrophotometry for concentration and purity (NanoDrop™ One Spectrophotometer, Thermo Scientific, Waltham, USA), and sequenced in both directions by Sanger method (3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Thereafter, sequences were subjected to BLASTn analysis (Altschul et al., 1990) for determining the identity with sequences deposited in GenBank database. Sequences were then aligned using MAFFT (Katoh et al., 2013), and improved on GUIDANCE2 (Sela et al., 2015). Best-fit evolutionary model was estimated as F81+I+G by jModeltest 2.1.4 (Darriba et al. 2012). The Bayesian Information Criterion (BIC) algorithm was used for phylogenetic inference and made by BEAST 1.8 with three independent runs of 50.000.000 MCMC. Trees were combined with TreeAnnotator 1.8 software and reconstruction was visualized with FigTree 1.4.0 software.

6.3 Results

All samples from both samplings successfully amplified the GAPDH gene and were submitted to *Mycoplasma* sp. detection. From the first set of sample collection, a total of five/8 (62,5%; CI: 3.05 – 8.63) rhesus monkeys tested positive for *Mycoplasma* spp. One sample that presented a strong band on electrophoresis was chose for sequencing and generated a 715 bp

sequence that was deposited in GenBank (XXXX.X). When analyzed by BLASTn the sequence showed similarity of 99% with '*Ca*. M. haemomacaque' sequences isolated from cynomolgus monkeys (*Macaca fascicularis*) in the United Stated (KC512401.1; AB820288.1).

Regarding the second sampling, three/6 (50%; CI: 1.87 – 8.12) animals tested positive for *Mycoplasma* spp. From these animals, one was negative in the first sampling. Two samples presenting strong bands on electrophoresis were chose for sequencing and generated sequences of 1.326 bp and 976 bp that were deposited in GenBank with accession number XXXX.X and XXXX.X, respectively.

In phylogeny analysis, our sequence formed a clade with '*Ca*. M. haemomacaque' isolates from USA (KC512401) and Japan (AB820288) within the *M. haemofelis* group (EU442639).

6.4 Discussion

Infection by *Mycoplasma* sp. was already described in rhesus monkeys (Peters et al., 1974). Based on partial 16S rRNA amplification and phylogeny, our results suggest that the species involved in the described infection is '*Ca*. M. haemomacaque'. This hemoplasma was recently proposed after its isolation in research cynomolgus monkeys (*Macaca fasicularis*) from USA through analysis of the 16S rDNA and partial RNase P genes (Maggi et al., 2013). Few later, this species was reported infecting captive *Macaca fuscata* from Japan with closely phylogenetic relationship with '*Ca*. M. haemomacaque' isolated in the USA (Maggi et al., 2013; Sashida et al., 2013).

A closely genotype was reported infecting free-range and captive monkeys from *Sapajus* genus in two different regions of northeastern Brazil and was suggested as a possible novel species of hemoplasma (Bonato et al., 2015; Ramalho et al., 2017). Otherwise, our study describes a genotype very close to '*Ca*. M. haemomacaque' from USA and Japan (Maggi et al., 2013; Sashida et al., 2013) infecting a research colony of monkeys in southeast Brazil.

Prevalence from the present study (62,5%) is lower when compared to studies that investigate hemoplasma infection in monkeys from research colonies out of Brazil, which ranged from 84.6% to 100% (Dillberger et al., 1974; Maggi et al., 2013; Sashida et al., 2014). However, it was high compared with studies conducted in Brazil with captive and free-range NHP that presented 35.7% and 25% rates (Bonato et al., 2015; Cubilla et al., 2017). Although it is more expected free-range animals to be infected, hemoplasma infection rates may be also high in colonies of laboratory animals (Conrado et al., 2015).

Regarding phylogenetic analysis, sequence 5 formed a robust clade together with '*Ca*. M. haemomacaque' isolates from USA and Japan (Maggi et al., 2013; Sashida et al., 2013), suggesting a greater proximity to this species. On the other hand, our sequence clurested distantly from *Mycoplasma* sp. genotypes isolated in *Sapajus* spp. monkeys from Brazil. Thus, a hypothesis that a potentially novel hemoplasma species circulates in NHP from northeastern region of Brazil, differently from the one isolated herein, may be raised. However, larger gene fragments and other genomic regions should be analyzed to confirm this statement.

Sequences from animals 2 and 4 formed a separately clade from animal 5 sequence and '*Ca*. M. haemomacaque' isolates from USA and Japan (Maggi et al., 2013; Sashida et al., 2013) clade, although it great presents post probabilities values. This found may suggest a homology from the sequences isolated in the present study and differences may be due sequencing proceeding.

Mycoplasma genotype close to '*Ca*. M. kahanei' is reported infecting *Alouatta* sp. monkeys from southern Brazil (Cubilla et al., 2017) and may be circulating in *Sapajus* monkeys from Brazilian Amazon (Bonato et al., 2015). Our phylogenetic analysis confirmed this relationship, however it clustered in the same group with *M. suis* (EU603330) whereas our *Mycoplasma* genotype was within the *M. haemofelis* group, suggesting that at least three genetically distinct hemoplasmas occur in NHP from Brazil.

Studies based on entire 16S rRNA gene and the intergenic spacer region between 16S and 23S rDNA genes showed that a monkey colony was entirely infected by the same strain of '*Ca*. M. haemomacaque' in Japan (Sashida et al., 2013). Although we obtained a high prevalence in our study (5/8; 62,5%), unfortunately it was not possible to sequencing more than one positive sample to identify diversity between the isolates.

Screening of research animals by hemoplasmas-PCR assays may be a desirable tool to avoid complications caused by infections (Hampel et al., 2014). Hematological or biochemical abnormalities were not associated to '*Ca*. M. haemomacaque' or other related genotypes in *Macaca fasicularis* and *Sapajus* sp. monkeys (Maggi et al., 2013; Ramalho et al., 2017). However, a moderate drop of erythrocytes may occur in splenectomised *Saimiri sciureus* monkeys (Contaim and Michel, 1999) and anemic episodes leading to obit were reported in M. fascicularis infected by *Mycoplasma* sp. (Dillberger et al., 1994).

Mycoplasma infection may lead to hematological changes (Hampel et al., 2014) and haptoglobin raising in apparently health hosts (Vilhena et al., 2018). Thus, outwardly "normal" animals may be not appropriate for researches because of unapparent infection effects.

Moreover, when infections are clinically visible, the use of drugs to clear the pathogens may influence on animal parameters and research results (Baker et al., 1999).

A late study regarding *M. coccoides* influence on viral infections showed that this hemoparasite species may influence rediculoendothelial system causing suppression of interferon response in rats (Glasgow et al., 1971). Once the animals sampled in the present study are used for arboviruses studies, it is important to investigate if hemoplasmas contamination may represent a bias in research development.

Transmission of *Mycoplasma* sp. agents in research groups of monkeys may be associated with needles or contaminated nasogastric tubes (Dillberger et al., 1994). Some *Mycoplasma* infections are associated with vectors transmissions, as ticks and fleas (Neirmark et al., 2004; Woods et al., 2005). Once the animals sampled herein received a monthly application of ivermectin, transmission may be associated with experimentational manipulation or direct contact between animals. Also, studies involving *M. muris* showed that latent infections may occur in laboratory animals with absence of ectoparasites (Bartelett and Pease, 1974). Transplacental transmission possibility is also concerned (Neimark et al., 2002). In all cases, it reveals the importance of ensure biosecurity proceedings and preventive screenings aiming to avoid contamination spread in research monkey colonies.

Moreover, some *Mycoplasma* species were described zoonotically infecting humans (Santos et al., 2008; Bosnoc et al., 2009; Sykes et al, 2010). Zoonotic potential should always be concerned to highlight possibilities of an occupational infection, especially for immunosuppressed persons.

6.5 Conclusion

Monkeys from the studied research colony are infected by *Mycoplasma* sp. More studies are necessary to elucidate the occurrence of hematological or clinical changes in infected animals and its transmission.

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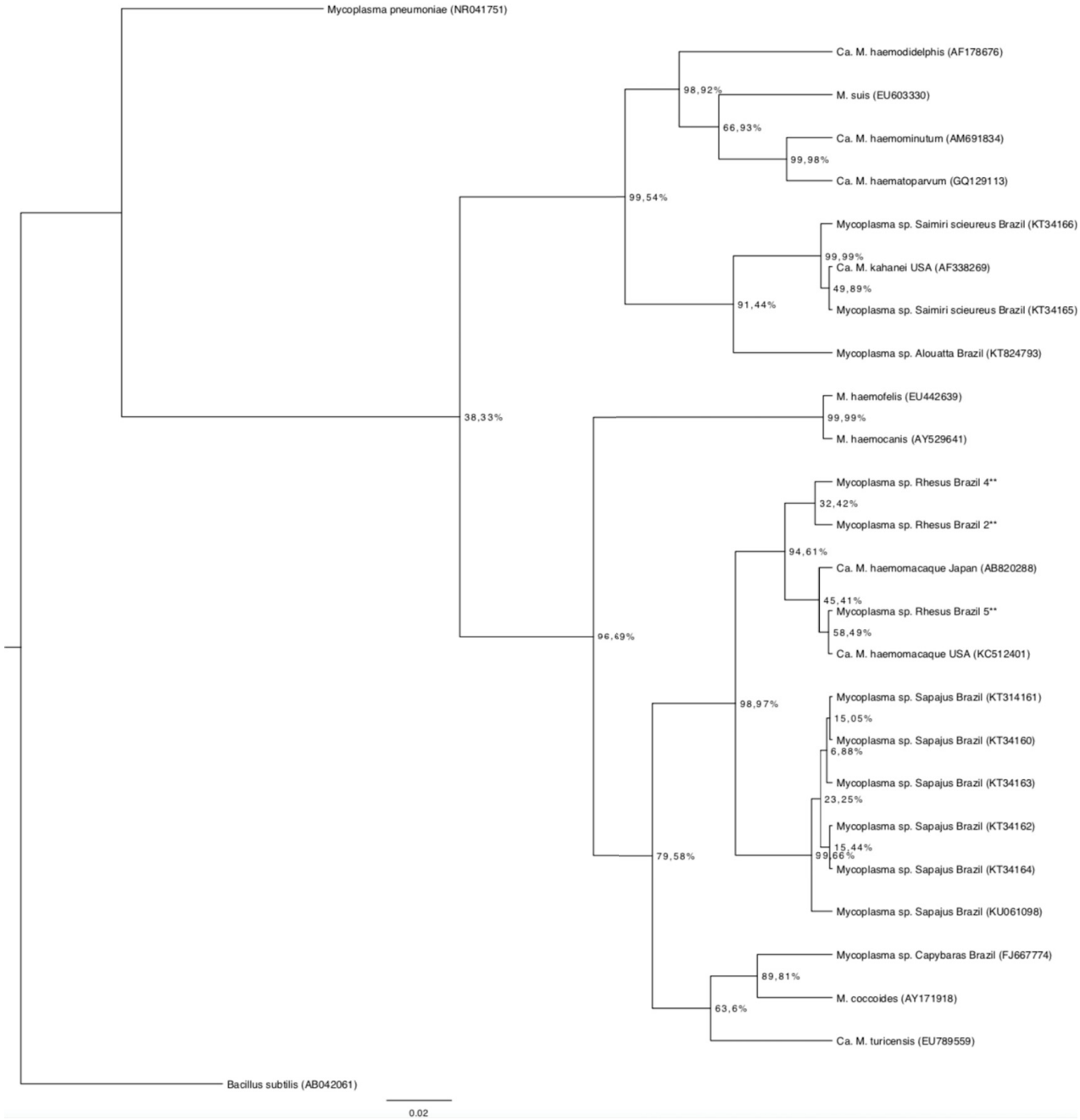

Figure 2. Phylogenetic tree based on partial 16S rDNA gene fragments of Mycoplasma sp. Tree was constructed by Bayesian Inference and a sequence from Bacillus subtillis was used as outgroup. Analysis were carried out applying the F81+I+G model and 1000 bootstrap replicates for all analyses. Our sequences are represented by ** after its name.

7 FINAL CONCLUSIONS

The clinical findings linked to anemia that were affecting the studied herd may be linked to infection by GI parasites. Sheep from the present study were parasitized by *R. microplus* ticks. The mean PCV concentration for sheep was 0.30 L/L. Nine out of 42 (21.42%; 95% CI: 11.71-35.94%) animals were anemic. Seven out of 42 (16.6%; 95% CI: 8.32–30.6%) animals were PCR-positive for *Mycoplasma* spp. None of the animals were positive for *Anaplasma* spp. and piroplasmids.

Twenty-four (63.15%; 95% CI: 47.2 – 76.6%) animals presented EPG values >500 and were considered positive for Strongylida type-eggs. All *Mycoplasma*-positive samples sequenced showed ≥99% identity with multiple *M. ovis* 16S rDNA gene sequences deposited in GenBank (accession nos. KU98374, KU983745, KU512718). The phylogeny showed two great clades dividing *Mycoplasma haemofelis* and *Mycoplasma suis* groups. Our sequences clustered together in a clade including *M. ovis* and *Mycoplasma* sp. sequences, inside the *M. suis* group. Polymorphism analysis revealed three different haplotypes from isolated sequences.

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